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Identification of immunogenic proteins within distinct molecular mass fractions of *Flavobacterium psychrophilum*

B R LaFrentz[1]*, S E LaPatra[2], D R Call[3], G D Wiens[4] and K D Cain[1,5]

1 Department of Fish and Wildlife Resources and the Aquaculture Research Institute, University of Idaho, Moscow, ID, USA

2 Clear Springs Foods Inc., Research Division, Buhl, ID, USA

3 Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA, USA

4 United States Department of Agriculture-Agricultural Research Service (USDA-ARS), National Center For Cool and Coldwater Aquaculture, Kearneysville, WV, USA

5 National Centre for Marine Conservation and Resource Sustainability, University of Tasmania, Launceston, Tasmania, Australia

Abstract

Flavobacterium psychrophilum is the aetiological agent of bacterial coldwater disease (CWD), and this pathogen has large economic impacts on salmonid aquaculture worldwide. Previously, it was demonstrated that high levels of protection against *F. psychrophilum* challenge were conferred to rainbow trout, *Oncorhynchus mykiss* (Walbaum), by immunization with distinct molecular mass fractions of the bacterium, and specific antibodies were correlated with protection. In this study, an immunoproteomic analysis of *F. psychrophilum* was performed using two-dimensional polyacrylamide gel electrophoresis and Western blotting with serum from fish immunized with high- and mid-molecular mass fractions of the bacterium. Mass spectrometry was used to determine the protein identity, and 15 immunogenic proteins were positively identified following Mascot searches of the *F. psychrophilum* genome. Based on known function and immunogenicity of homologous proteins in other bacterial pathogens, antibodies specific for several of the identified proteins may be important for protective immunity from CWD. These include outer membrane protein OmpA (P60), trigger factor, ClpB, elongation factor G, gliding motility protein GldN and a conserved hypothetical protein. This work increases the understanding of the protective humoral immune response of rainbow trout against these distinct molecular mass fractions of *F. psychrophilum* and provides new potential targets for recombinant protein vaccine development.

Keywords: bacterial coldwater disease, *Flavobacterium psychrophilum*, immunoproteomic, vaccine.

Correspondence *Dr K Cain, Department of Fish and Wildlife Resources and the Aquaculture Research Institute, University of Idaho, PO Box 441136, Moscow, ID 83844-1136, USA (e-mail: kcain@uidaho.edu)*

**Present address: USDA-ARS, Aquatic Animal Health Research Unit, 990 Wire Road, Auburn, AL 36832-4352, USA.*

Introduction

Flavobacterium psychrophilum is a Gram-negative bacterial fish pathogen that causes bacterial coldwater disease (CWD) and rainbow trout fry syndrome. Preventative measures include the use of management strategies to reduce risk factors such as stress, poor water quality and cutaneous lesions (Groff & LaPatra 2000). Even with these measures in place, mortality related to *F. psychrophilum* infections commonly occurs and generally requires treatment. Management and treatment options include reducing pathogen concentrations, eliminating the spread of the pathogen (Bebak, Welch, Starliper, Baya & Garner 2007) and application of antibiotics. The effectiveness of such treatments is usually inconsistent; therefore, a vaccine to prevent CWD is desired, but none are commercially available.

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Protective immunity against CWD is mediated, in part, by specific antibodies (LaFrentz, LaPatra, Jones & Cain 2003), and this knowledge has prompted research to better understand the humoral immune response and identify immunogenic components of *F. psychrophilum* as targets for subunit or recombinant protein vaccine development. Fish generate specific antibody responses against protein and carbohydrate components of the bacterium (Crump, Perry, Clouthier & Kay 2001; LaFrentz, LaPatra, Jones & Cain 2004; LaFrentz, Lindstrom, LaPatra, Call & Cain 2007; Sudheesh, LaFrentz, Call, Siems, LaPatra, Wiens & Cain 2007; Högfors, Pullinen, Madetoja & Wiklund 2008), and some studies have definitively identified some of these components (Crump, Burian, Allen & Kay 2005; Dumetz, Duchaud, LaPatra, Le Marrec, Claverol, Urdaci & Le Henaff 2006; Crump, Burian, Allen, Gale & Kay 2007; Dumetz, LaPatra, Duchaud, Claverol & Le Henaff 2007; LaFrentz *et al.* 2007; Sudheesh *et al.* 2007; Dumetz, Duchaud, Claverol, Orieux, Papillon, Lapaillerie & Le Henaff 2008). Although knowledge has been gained of the immunogenic components of *F. psychrophilum*, only two potential protective antigens have been identified. Massias, Dumetz, Urdaci & Le Henaff (2004) identified and characterized an 18 kDa OmpH-like protein and subsequently tested its ability to elicit protective immune responses in rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Dumetz *et al.* 2006). Specific antibodies against *F. psychrophilum* were induced following injection immunization with the semi-purified protein emulsified with an adjuvant and protective immunity was conferred. Crump *et al.* (2007) identified an immunoreactive ribosomal L10-like protein of *F. psychrophilum* by screening an expression library with rabbit antiserum. The protein was expressed in *Escherichia coli* as a fusion protein and was shown to induce a protective immune response in rainbow trout following injection immunization with the protein incorporated with an adjuvant. These studies demonstrate the potential for the development of a vaccine for CWD based on semi-purified or recombinant proteins.

It was previously demonstrated that immunization of rainbow trout with distinct molecular mass fractions of *F. psychrophilum* induced protective immune responses when incorporated with an adjuvant (LaFrentz *et al.* 2004). Immunization of fish with high- and mid-molecular mass fractions resulted in high levels of protection following challenge with relative percent survivals (RPS) of 94% and 58%, respectively. The results demonstrated that antibodies were involved in the protection observed, and Western blot analyses using single-dimension electrophoresis demonstrated that fish immunized with both fractions exhibited antibodies specific for protein antigens. The goal of this study was to identify proteins of *F. psychrophilum* that were immunogenic in rainbow trout immunized with the high- and mid-molecular mass fractions to increase the understanding of the protective humoral immune response against these regions.

Materials and methods

Bacterial culture and preparation of whole-cell lysate

A virulent isolate of *F. psychrophilum*, CSF-259-93 (Sudheesh *et al.* 2007), was used in this study. Stock suspensions of the strain were maintained at −80 °C in 20% glycerol and were used to inoculate cultures. *Flavobacterium psychrophilum* was cultured in tryptone yeast extract salts, and whole-cell lysates were prepared as described by LaFrentz, LaPatra, Call, Wiens & Cain (2009). Lysates were stored at −80 °C until needed for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

2D-PAGE and Western blot analysis

Whole-cell lysate proteins from *F. psychrophilum* were diluted in rehydration buffer, and immobilized pH gradient (IPG) strips (7 cm, pH 4–7; Bio-Rad) were passively rehydrated with 60 μg of protein and were subjected to isoelectric focusing as described by LaFrentz *et al.* (2009). IPG strips were equilibrated and second-dimension separation was performed using 10–20% linear gradient polyacrylamide gels (Bio-Rad) as described by LaFrentz *et al.* (2009). Precision Plus (Bio-Rad) protein standards were included for estimation of the molecular masses of protein spots. Gels were either stained with SYPRO Ruby (Bio-Rad) and digitally imaged using a FLUOR-S Multi-Imager (Bio-Rad) or used in Western blot analyses.

Western blot analyses were performed to identify proteins recognized by serum antibodies from fish immunized with high- and mid-molecular mass fractions of *F. psychrophilum*. Archived (−80 °C)

serum samples obtained in the experiments conducted by LaFrentz *et al.* (2004) were used in this study. A single pool of serum from each group was prepared by combining all remaining serum samples and used for Western blotting. This consisted of combining equal volumes of previously pooled (three fish per pool) serum samples with titres ranging from 51 200 to 102 400 and from 6400 to 51 200 for the high- and mid-molecular mass fractions (LaFrentz *et al.* 2004), respectively. Additionally, serum samples from fish immunized with adjuvant alone (LaFrentz *et al.* 2004) were included as negative controls. Proteins were transferred to nitrocellulose membranes, and immunogenic proteins were identified by Western blot analysis as previously described (LaFrentz *et al.* 2004).

Identification of immunogenic proteins

Following Western blot analyses, immunogenic proteins were matched to the corresponding proteins in Sypro Ruby-stained gels and then manually excised. Excised proteins were placed in 5% acetic acid and submitted to the Proteomics Core Facility at Michigan State University (East Lansing, MI, USA) for identification by LC-MS/MS analysis and searches against the *F. psychrophilum* (ATCC 49511) genome (Duchaud, Boussaha, Loux, Bernardet, Michel, Kerouault, Mondot, Nicolas, Bossy, Caron, Bessieres, Gibrat, Claverol, Dumetz, Le Henaff & Benmansour 2007), as previously described (LaFrentz *et al.* 2009). Estimation of the theoretical molecular mass (M_r) and isoelectric points (pI) of the identified proteins was determined using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). For each identified protein, the amino acid sequences from the published *F. psychrophilum* (ATCC 49511) genome were compared with the CSF-259-93 genome (G. Wiens, unpublished) to determine the percent identity.

Results and discussion

An immunoproteomic approach was used to identify proteins of *F. psychrophilum* that were immunogenic in rainbow trout immunized with the high- and mid-molecular mass fractions. These fractions were referred to as 70–100 kDa and 41–49 kDa, because those were the targeted size ranges for fractionation; however, the actual size ranges of proteins isolated corresponds to approximately 45–100 kDa and 30–45 kDa, respectively (LaFrentz *et al.* 2004). Rainbow trout immunized with the high-molecular mass fraction exhibited antibodies specific for numerous proteins of varying levels of intensity with molecular masses ranging from approximately 31–100 kDa (Fig. 1a). The majority of the immunogenic proteins had molecular masses of 45–100 kDa, which is the approximate size range of the proteins used for immunization. Fish immunized with the mid-molecular mass fraction exhibited antibodies specific for proteins of varying levels of intensity with approximate molecular masses of 16–100 kDa (Fig. 1b). This was unexpected, as these fish were immunized with proteins of approximate molecular masses of 30–45 kDa. This may be explained by the possibility that these fish generated specific antibodies for low quantities of contaminating protein (greater and lesser than 30–45 kDa) that was observed in the isolated protein fraction used for immunization (LaFrentz *et al.* 2004) or the anti-

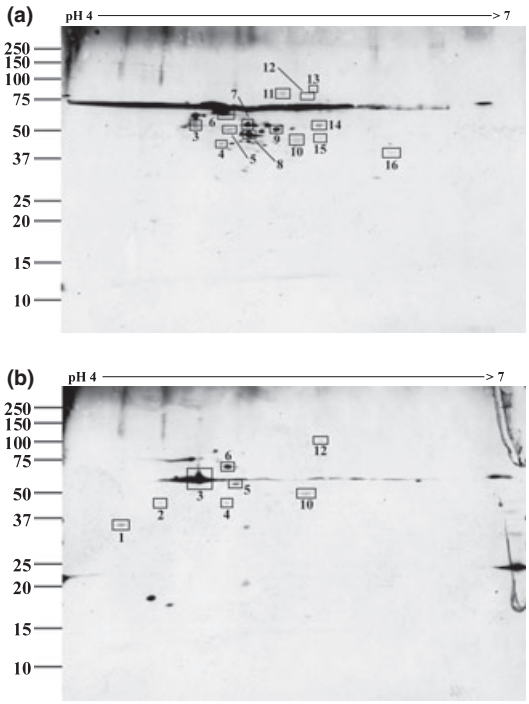

Figure 1 2D-PAGE–Western blot of whole-cell lysate proteins of *Flavobacterium psychrophilum*. Proteins were probed with serum antibodies from rainbow trout immunized with (a) high-molecular mass and (b) mid-molecular mass fractions of the bacterium. Protein spot numbers indicate immunogenic proteins that could be confidently matched to stained gels (Fig. 2; Table 1). Molecular mass markers (kDa) are indicated to the left of each Western blot.

bodies reacted with breakdown products or multimers of the immunogenic proteins. Control serum from fish immunized with adjuvant alone exhibited weak reactivity to a few protein spots (data not shown), consistent with single-dimension Western blots previously performed (LaFrentz *et al.* 2004).

Western blots were compared with stained gels to match immunogenic protein spots to the corresponding protein spot in the stained gels for excision. There were a number of protein spots that were immunoreactive, but could not be matched to a corresponding protein in stained gels (Figs 1 & 2). This was most likely due to the higher detection sensitivity of Western blots compared with Sypro Ruby staining. Nevertheless, 16 could be confidently matched to the corresponding protein spot (Fig. 2) and were excised and submitted for identification by LC-MS/MS (Table 1). Single proteins were positively identified in each protein spot submitted with the exception of protein spot nos 5 and 9. Two proteins were positively identified in protein spot no. 5, and analysis of protein spot no. 9 resulted in no identification. Two of the protein spots (protein spot nos 1 and 3) were identified as the same protein. Thus, a total of 15 individual immunogenic proteins were identified. The amino acid sequences of each identified protein were 100% identical between the *F. psychrophilum* ATCC 49511 and CSF-259-93 genomes. The identified immunogenic proteins are members of diverse functional groups (Table 1).

Characteristics of protective proteins for extracellular pathogens, such as *F. psychrophilum*, include the ability to elicit a specific antibody response, cell surface exposure and possible roles in virulence. These are important because it is generally accepted that antibodies specific for extracellular pathogens are important for protection owing to effector mechanisms such as neutralization/bactericidal activity, opsonization, agglutination and activation of the complement cascade. Similarly, antibody has been demonstrated to be an important host factor for protection against *F. psychrophilum* (LaFrentz *et al.* 2003). Some of the proteins identified in this study have been shown to exhibit these characteristics, and this suggests that antibodies specific for them are involved in protective immunity against CWD.

The OmpA of *F. psychrophilum* has previously been characterized (Merle, Faure, Urdaci & Le Henaff 2003; Dumetz *et al.* 2007) and demonstrated to be immunogenic and surface exposed (Dumetz *et al.* 2007, 2008). In general, the OmpA family of proteins tend to be highly immunogenic (Puohiniemi, Karvonen, Vuopio-Varkila, Muotiala, Helander & Sarvas 1990; Mahasreshti, Murphy, Wyckoff, Farmer, Hancock & Confer 1997), and there is evidence to suggest that these proteins may be virulence factors with roles in colonization and adhesion (Prasadarao, Wass, Weiser, Stins, Huang & Kim 1996; Serino, Nesta, Leuzzi, Fontana, Monaci, Mocca, Cartocci, Masignani, Jerse, Rappuoli & Pizza 2007). Recombinant OmpA proteins from different bacterial pathogens have been produced and tested as vaccine candidates with some success (von Specht, Knapp, Muth, Broker, Hungerer, Diehl, Massarrat, Seemann & Domdey 1995; Baumann, Mansouri & von Specht 2004). Similarly, a preliminary study with rainbow trout indicated that immunization with semi-purified OmpA of *F. psychrophilum* induced a humoral immune response that conferred some protection following CWD challenge (Dumetz *et al.* 2007). The identification of the OmpA protein as immunogenic in this study provides further support for a protective role of antibodies directed against this protein.

Two chaperone proteins, trigger factor and ClpB, and the elongation factor G (EF-G) of *F. psychrophilum* were identified as immunogenic. Each of these proteins are immunogenic (Havlasova, Hernychova, Brychta, Hubalek, Lenco, Larsson, Lundqvist, Forsman, Krocova, Stulik & Macela 2005; Boonjakuakul, Gerns, Chen, Hicks, Minnick, Dixon, Hall & Koehler 2007), and trigger

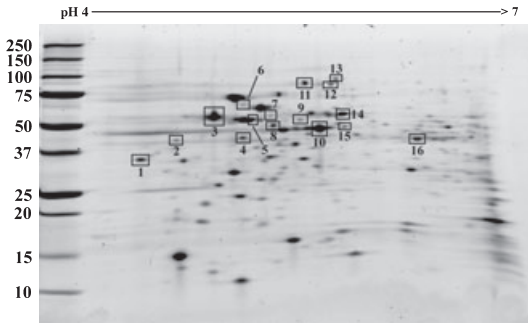

Figure 2 2D-PAGE analysis of whole-cell lysate proteins of *Flavobacterium psychrophilum*. Proteins were visualized by Sypro Ruby staining. Protein spot numbers correspond to immunogenic proteins in Fig. 1 and were identified by LC-MS/MS (Table 1). Molecular mass markers (kDa) are indicated to the left of the gel.

Table 1 Immunogenic proteins of *Flavobacterium psychrophilum* identified by LC-MS/MS. Protein spot numbers refer to proteins in Figs 1 & 2. Superscripts adjacent to spot numbers indicate immunoreactivity in serum from rainbow trout immunized with (a) high- or (b) mid-molecular mass fractions of *F. psychrophilum*

Spot no.	Identified protein	Functional group	Reference sequence	Theoretical M_r (kDa)/pI	No. peptides matched	Sequence coverage (%)	Mascot score
1[b]	OmpA family outer membrane protein P60	Cell wall	YP_001295092	49.4/4.87	7	20	796
2[b]	Hypothetical protein FP1439	Protein of unknown function	YP_001296324	37.2/4.73	2	7	128
3[a,b]	OmpA family outer membrane protein P60	Cell wall	YP_001295092	49.4/4.87	19	43	1664
4[a,b]	Gliding motility protein GldN	Mobility and chemotaxis	YP_001296836	39.1/5.82	12	36	928
5[a,b]	Trigger factor (TF)	Protein folding	YP_001296950	49.9/4.88	19	39	1142
	F0F1 ATP synthase subunit beta	Membrane bioenergetics	YP_001295052	54.4/4.9	7	17	527
6[a,b]	Pyruvate dehydrogenase E2 component	Main glycolytic pathway	YP_001296276	57.9/4.91	3	8	360
7[a]	Cysteine desulphurase activator complex subunit SufB	Metabolism of sulphur	YP_001297249	53.8/5.0	2	5	91
8[a]	Molecular chaperone DnaK	Protein folding	YP_001295780	67.3/4.83	6	8	331
9[a]	No identification	–	–	–	–	–	–
10[a,b]	Elongation factor TU	Translation elongation	YP_001296081	43.2/5.14	19	56	1418
11[a]	Elongation factor G	Translation elongation	YP_001296235	79.3/5.12	16	25	947
12[a,b]	Aconitate hydratase	TCA cycle	YP_001296062	81.1/5.31	12	23	715
13[a]	ATPase with chaperone activity ATP-binding subunit (ClpB)	Adaptation to atypical conditions	YP_001296639	96.9/5.33	2	2	115
14[a]	F0F1 ATP synthase subunit alpha	Membrane bioenergetics	YP_001297312	56.5/5.36	15	29	1146
15[a]	S-adenosylmethionine synthetase	Metabolism of amino acids and related molecules	YP_001295202	45.6/5.38	7	20	289
16[a]	Valine dehydrogenase[1]	Metabolism of amino acids and related molecules	YP_001295457	39.8/5.68	11	39	1239

M_r, molecular mass; pI, isoelectric point.
[1]Annotated as a leucine dehydrogenase in the CSF-259-93 genome (G. Wiens, unpublished).

factor and ClpB are putative virulence factors (Chastanet, Derre, Nair & Msadek 2004; Bigot, Botton, Dubail & Charbit 2006) in other bacterial species. Similarly, LaFrentz *et al.* (2009) demonstrated that the ClpB protein of *F. psychrophilum* is upregulated *in vivo* and may have a role in pathogenesis. Trigger factor and EF-G can be isolated from the outer membranes of some bacterial pathogens (Rhomberg, Karlberg, Mini, Zimny-Arndt, Wickenberg, Rottgen, Jungblut, Jeno, Andersson & Dehio 2004; Ebanks, Goguen, McKinnon, Pinto & Ross 2005; Boonjakuakul *et al.* 2007). The role of specific antibodies against ClpB and EF-G in protective immunity has not been reported, but research has demonstrated that immunization of mice with the trigger factor protein of *Brucella melitensis* induces a humoral immune response and protective immunity (Yang, Hudson, Walters, Bargatze & Pascual 2005; Yang, Walters, Robison, Trunkle & Pascual 2007).

The GldN protein of *F. psychrophilum* was identified as immunogenic in this study and is one of the many proteins involved in the gliding motility of *Flavobacterium* spp. In *F. johnsoniae*, the GldN protein was predicted to localize to the cell envelope (Braun, Khubbar, Saffarini & McBride 2005). The role of gliding motility in the pathogenesis of *F. psychrophilum* is not understood, but it may play an important role in colonization and movement within the host. LaFrentz *et al.* (2009) demonstrated an upregulation of this protein following growth of the bacterium *in vivo* and under iron-limited conditions and suggested that it has a role in pathogenesis. Further, Martinez, Casado & Enriquez (2004) suggested that *F. psychrophilum* uses a combination of proteolytic activity and gliding motility to gain entry into fin tissue. These observations suggest that antibodies specific for GldN may prevent effective motility of *F. psychrophilum* and enhance protection following infection.

A hypothetical protein (protein spot no. 2; FP1439) was identified as immunogenic in the present study. The protein exhibits similarity to a periplasmic *E. coli* FKBP-type chaperone protein (Dumetz *et al.* 2008). Dumetz *et al.* (2008) also identified this protein as immunogenic, and it was found in an outer membrane extract of *F. psychrophilum*. The function and cellular location of this protein warrants further investigation, as antibodies directed against it may be important for protection from CWD.

The chaperone protein DnaK, elongation factor TU (EF-TU), ATP synthase and SufB of *F. psychrophilum* were identified as immunogenic in the present study. Investigations of homologous proteins in other bacterial species have indicated that these may be important for protective immunity (reviewed in Plant, LaPatra & Cain 2009; Plant, LaPatra, Call & Cain 2011). Therefore, research was pursued to test the hypothesis that immunization of rainbow trout with these proteins will elicit humoral immune responses and provide protection against *F. psychrophilum* challenge. Immunization with recombinant DnaK induced significant specific antibody responses against the protein, but no protection was observed following *F. psychrophilum* challenge (Plant *et al.* 2009). Immunization with recombinant EF-TU, ATP synthase and SufB failed to induce significant specific antibody responses, and no protection was observed following bacterial challenge (Plant *et al.* 2011). These studies suggest that these recombinant proteins from *F. psychrophilum* are not protective when administered independently with adjuvant via intraperitoneal injection. Nevertheless, these proteins appear to be important for protective immunity in other pathogenic bacteria because of surface exposure and/or putative roles in virulence. It is not known whether these protein homologues from *F. psychrophilum* share these characteristics, and it is likely that specific antibodies against multiple protective proteins are necessary to obtain protective immunity from CWD.

LaFrentz *et al.* (2004) suggested antibody-mediated protection in rainbow trout immunized with the high- and mid-molecular mass fractions, and challenge of these fish with *F. psychrophilum* resulted in RPS values of 94% and 58%, respectively. The results of the present study increased the understanding of the humoral immune response against these distinct fractions of *F. psychrophilum* by identifying the specificity of the protective antibodies. Fish immunized with the high-molecular mass fraction exhibited antibodies specific for proteins as well as carbohydrates (LaFrentz *et al.* 2004), which were subsequently identified and characterized as components of the glycocalyx (LaFrentz *et al.* 2007). Thus, the combination of antibodies specific for the glycocalyx and the proteins identified in the present study mediated the robust protection observed. Fish immunized with the mid-molecular fraction exhibited antibodies specific only for proteins of *F. psychrophilum*

(LaFrentz *et al.* 2004), and therefore, the proteins identified in the present study using this serum likely mediated the moderate level of protection observed.

In summary, 15 proteins of *F. psychrophilum* were identified by immunoproteomics using high-titre serum antibodies from rainbow trout that were immunized with distinct molecular mass fractions. Based on research conducted on homologous proteins in other bacterial pathogens, antibodies specific for OmpA, trigger factor, ClpB, EF-G, GldN and a hypothetical protein may have been involved in the protection observed. The results also suggest that antibodies specific for DnaK, EF-TU, ATP synthase and SufB have a role in protective immunity, although subsequent immunization studies with recombinant proteins failed to demonstrate this. The remaining proteins identified in this study have not been previously demonstrated to be immunogenic in other bacterial pathogens, but may represent important proteins for immunity against *F. psychrophilum*. This work increases the understanding of the protective humoral immune response of rainbow trout against these distinct molecular mass fractions of *F. psychrophilum* and provides new potential targets for recombinant protein vaccine development.

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